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Effect of Culture Medium Supplementation with b-mercaptoethanol and Amino Acid on Canine Intergeneric Embryo Development with Porcine Oocyte Cytoplasm Recipient

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Abstract

The present study investigated the effect of culture medium supplementation with b-mercaptoethanol (b-ME) and amino acid (AA) on canine intergeneric embryo development with porcine oocyte cytoplasm. Porcine cumulus oocyte complexes (COCs) were collected from slaughterhouse and matured in TCM-199 supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 75 mg/l kanamycin, 10 ng/ml epidermal growth factor, equine chorionic gonadotropin (eCG), 10 IU/ml human chorionic gonadotropin (hCG), and 10% (v/v) porcine follicular fluid (pFF) at 39 °C in a humidified atmosphere of 5% CO_2 for 42-44 h and donor cell collected from ear skin afghanhound male dog. After somatic cell nuclear transfer (SCNT), embryo development were examined for cleavage rate and 144 hr for final development after cultured in media. The result shows that, amino acid and b-mercapoethanol addition in culture medium (NCSU-23) have no effect on embryo development. The development rate of embryo until 16 cell stage in NCSU and NCSU supplement are 4.67% and morula stage are 3.73% and 4.67%.

Key words : intergeneric clone embryo, canine, b-mercaptoethanol (b-ME), amino acid (AA)

Introduction

Interspecies somatic cell nuclear transfer method was firstly applied for conservation of endangered animals. The highly publicized that an adult sheep had been cloned from the nucleus of a frozen somatic cell (Wilmut *et al*., 1997) speculated that cloning technologies might be applied to increase population sizes of endangered species, or even restore them following extinction (Cohen, 1997; Wen *et al*., 2005).

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Interspecies nuclear transfer also provides a possible approach to clone animal species whose oocytes were difficult to obtain (Jumnian *et al*., 2002; Wen *et al*., 2003). Several studies have shown that oocyte cytoplasm from bovine, rabbits and sheep can support early development of embryos produced by nuclear transfer of somatic cells nuclei from various mammalian species (Dominko *et al*., 1999; White *et al*., 1999; Cohen *et al*., 1999; Lanza *et al*., 1999; Chen *et al*., 1999; 2002; Wen *et al*., 2003). Recently, the successes of cloning gaur (Lanza *et al*., 2000) and mouflon (Loi *et al*., 2001) have demonstrated that the technique of interspecies cloning can be practically applied to save highly endangered species, such as the giant panda, Ovis orientalis musilmon, buffalo, bos gaurus (White *et al*.,

1999; Lanza *et al*., 2000; Vogel *et al*., 2001; Chen *et al*., 2002; Jumnian *et al*., 2002; Sansisena *et al*., 2005).

The establishment of optimum culture medium for embryos of domestic animals derived from in vitro fertilized (IVF) and somatic cell nuclear transfer (SCNT) is very critical for applied as well as basic research. Many factors are known to influence the in vitro culture (IVC) of mammalian embryos. Among them, media composition, culture atmosphere, temperature, oxygen tension, osmotic pressure, free radical scavengers, volume of culture drops, and embryo manipulation such as microinjection and cryopreservation are reported to be influence embryo development and quality (Quinn and Harlow, 1978; Harlow and Quinn, 1982; Umaoka *et al*., 1992; Kooyman and Pinkert, 1994; Bagis *et al*., 2002; Bagis and Odaman, 2004). Factors that have a negative impact on the in vitro development of the embryo include oxidative stress and composition of culture medium (Umaoka *et al*., 1992).

Amino acids serve a variety of physiological functions, including: the synthesis of proteins and nucleotides (Epstein and Smith 1973; Alexiou and Leese 1992; Katchadourian *et al*., 1994), nutrition and energy provision (Lane and Gardner, 1997; 1998; Houghton *et al*., 2002), osmoregulation (Van Winkle and Campione, 1996; Dumoulin *et al*., 1997; Dawson et al., 1998), protection against oxidative stress (Lindenbaum 1973; Nasr-Esfahani *et al*., 1992), pH regulation (Bavister and McKiernan, 1993; Edward *et al*., 1998), signalling molecule biosynthesis (Wu and Morris, 1998), trophectoderm differentiation (Martin and Sutherland, 2001) and basement membrane formation between primitive endoderm and ectoderm (Biggers *et al*., 2000). Although achievements have made early stages of feasible embryonic development in vitro, the adequate culture conditions for the preimplantation porcine embryo have yet to be determined. Differential developmental competence in response to various culture media has been demonstrated between IVF and SCNT embryos (Chung *et al*., 2002). Four-cell developmental block was overcome by using NCSU-23 in vivo porcine oocytes (Machaty *et al*., 1998).

Glutathione is the major non-protein sulfidryl compound present in mammalian cells. Multiple actions have been described for GSH, including increasing amino acid transport, stimulating DNA and protein synthesis, reduction of disulfides and protection against toxic effects of oxidative damage (Meister, 1983; Lafleur *et al*., 1994). It has been demonstrated that adding beta mercaptoethanol (b-ME) increased intracellular GSH in the mo use lymphocytes (Zmuda and Friedenson, 1983). In mouse embryo, it was reported that glutathione content decreases about tenfold during preimplantation development and later stage embryos would be more sensitive to oxidative stress because of their lower GSH content (Nasr-Esfahani and Johnson, 1992). As for embryotrophic effect of b-ME in embryos, Takahashi *et al*. (1993) demonstrated that the adding low molecular weight thiols such â-ME and cysteamine into culture medium enhanced cysteine mediated GSH synthesis and improved the production of 6- to 8-cell bovine embryos in vitro. In addition to this, increased intracellular GSH content in oocytes and embryos of varied developmental stages improves embryonic development and embryo quality, resulting in higher blastocyst yield (Takahashi *et al*., 1993). The results obtained by Bagis and Odaman (2004a) demonstrated that the combined treatment of b-ME and amino acid to 1-cell stage embryos not only enhanced in vitro development to the blastocyst stage but also improved both the number of blastocyst

cells and live fetuses in mouse.

Although studies have reported using mouse embryos, in intergeneric canine cellnuclear transfer embryo culture, the effect of b-ME and AA supplement in culture medium on embryo development is largely not known. Therefore, the present study was conducted to examine the efficacy of NCSU-23 medium supplemented with b-ME and AA on the developmental competence of intergeneric canine embryo using porcine oocyte in order to improve in vitro culture conditions.

Materials and Methods

Collection of porcine oocytes and invitro maturation (IVM)

Ovaries were obtained from a local abattoir and transported to the laboratory in physiological saline at 30 to 35°C. Antral follicles 3 to 6 mm in diameter were aspirated using an 18-gauge needle attached to a 5-ml disposable syringe. Cumulus-oocyte complexes (COCs) with compact cumulus cells were collected from the aspirate and washed several times in HEPES-buffered tissue culture medium (TCM)-199 (Life Technologies, Rockville, MD, USA). The COCs were then placed in IVM medium (Earle's salts- and L-glutamine-containing TCM-199 supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 75 mg/l kanamycin, 10 ng/ml epidermal growth factor (Sigma-Aldrich), equine chorionic gonadotropin (eCG, Intervet, Boxmeer, Netherland), 10 IU/ml human chorionic gonadotropin (hCG, Intervet Boxmeer Netherland), and 10% (v/v) porcine follicular fluid (pFF). The pFF was aspirated from superficial antral follicles 8 to 10 mm in diameter from prepubertal gilts. After centrifugation at 1,600 x g for 30 min, supernatant was collected and filtered sequentially through 1.2 mm and 0.45 mm syringe filters (Gelman Sciences, Ann Arbor, MI, USA). Prepared pFF was then stored at -20°C until use.

A group of 50 COCs was cultured in 500-ml IVM medium at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 hr, COCs were transferred to eCG- and hCG-free IVM medium and cultured further for 20-22 h. At the end of the culture, oocytes were freed from cumulus cells by repeated pipetting in IVM medium containing 0.1 % hyaluronidase. Oocytes with a first polar body, intact zona pellucida, evenly granulated cytoplasm, expanded cumulus cells and distinct ooplasmic membrane were provided for SCNT of this study.

Preparation of recipient oocytes for somatic cell nuclear transfer

After 42-44 h of maturation, the oocytes were freed from cumulus cell by pipetting in HEPES-buffered NCSU-23 medium supplemented with 0.1% hyaluronidase. Oocytes were cultured in NCSU-23 containing 5 mg/ml bisbenzimide (Hoechst 33342; Sigma-aldrich Co.) and 7.5 mg/ml cytochalasin B for 30 min. Oocytes were placed in a 4 ml drop of HEPES-buffered NCSU-23 medium on working dishes. Each recipient oocyte was held with a holding micropipette (110 µm in outer and 24 µm in inner diameter) and zona pellucida was partially dissected with a fine glass needle to create a slit near the polar body. Then, the first polar body and adjacent cytoplasm containing metaphase plate were removed by squeezing. Enucleated oocyte were visually verified by ultraviolet fluorescence, keeping exposure to a minimum. The enucleated oocytes were then placed in NCSU23-D (Table 7) and used for SCNT.

Injection, Electrofusion and activation

Injection was performed in 4 ml drop of HEPES-buffered NCSU23-W medium and covered with light mineral oil. A single cell

with smooth membrane was transferred into the perivitelline space of an enucleated oocyte. Before nuclear transfer, transfected donor cell were identified emission of greenfluorescence under an epifluorescent microscope using a standard fluorescein isothiocyanate (FITC) filter set. Round-shaped and green-colored small cell were individually injected into perivitelline space of enucleated oocytes.

Reconstructed oocytes were fused and activated simultaneously. The reconstructed oocytes were equilibrated for 10 sec in fusion medium (0.26 M mannitol, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mM HEPES and 0.05% BSA, Table 3) and transfer to a fusion chamber with two electrodes (3.2 mm gap, BTX Inc., San Diego, CA) overlaid with the mannitol medium. Reconstructed oocytes (5-7 oocytes) were aligned with a fine mouth-controlled Pasteur pipette in parallel with a fusion chamber.

Fusion was induced with a single DC pulse of 1.86 kV / cm for 30, by on a Electro-cell Manipulator 2001 (BTX Inc.). All treated oocytes were washed three times with NCSU23-W supplemented with 4 mg / ml BSA, placed in 25 ml microdrops (10-15 oocytes per drop) of NCSU23-D under mineral oil and cultured at 39°C, 5% CO_2, 5% O_2 and 90% N_2. Fused oocytes were determined one hour after the electrical pulse under microscope.

Subsequent culture

The reconstructed embryos were cultured in 25 ml drops of NCSU23-D overlaid with mineral oil at 39ºC in humidified 5% CO_2, 5% O_2 and 90% N_2 atmosphere. Ten to fifteen embryos were cultured together. Cleavage rate was recorded after 48 h post fusion. On 7 days post fusion, the development of reconstructed embryos was recorded, and GFP expression rate in embryo was examined under FITC filter.

Preparation of donor canine cells

Canine fibroblast cells were isolated from ear skin. The external surface of canine ear skin was shaved and cleaned aseptically. A piece of ear skin tissue about 100 mm^3 wide and 2 mm thick was biobsied and immediately immersed in D-PBS (Life technologies). After washing, the tissues were minced by a surgical blade on a 100 mm culture dish, followed by dissociation by 0.25% (w / v) trypsin containing 1 mM EDTA for 1 to 2 h at 38°C. Trypsinized cells were washed once by centrifugation (300xg, 2 min) and subsequently seeded into 100 mm culture dishes and cultured for 6-8 day in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v / v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 % (v / v) non-essential amino acid and 10 ml / ml penicillin / streptomycin solution in a humidified atmosphere of 95% air, 5% CO_2 at 38°C before removal of unattached clumps of cells or explants. The attached cells were passage by trypsinization when confluent.

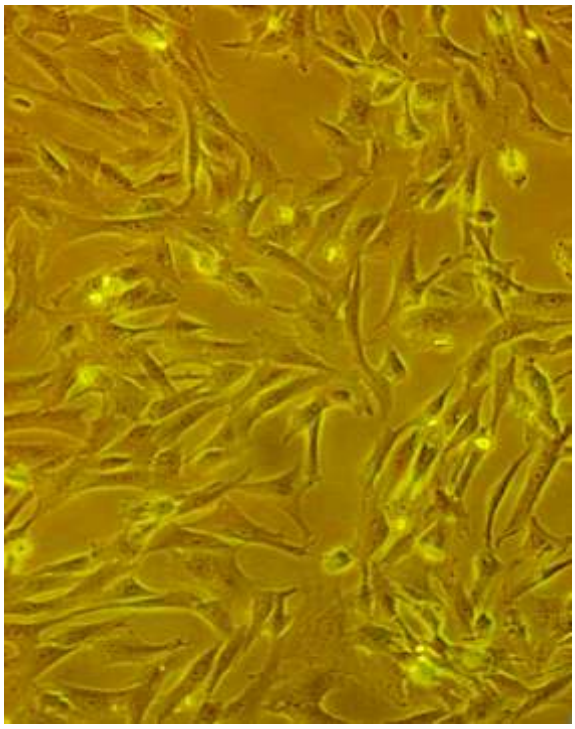

Figure 1. Adult canine ear skin fibroblast cell line

Statistical analysis

Data from all experiment in this experiment were analyzed using the statistical Analysis System (SAS) program. Data were subjected to analysis of variance (ANOVA) and protected least significant different (LSD) test to determine differences among experimental groups. When a significant model effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was determined where P value was less than 0.05.

Table 1. Composition of North Carolina State University (NCSU)-23 medium

Ingredient	NCSU23-W (mmol/L)	NCSU23-D (mmol/L)
NaCl	108.73	108.73
KCl	4.78	4.78
$CaCl_2$	1.70	1.70
KH_2PO_4	1.19	1.19
$MgSO_4 \cdot 7H_2O$	1.19	1.19
$NaHCO_3$	4.01	25.07
Glucose	5.55	5.55
Glutamine	1.00	1.00
Taurine	-	7.00
Hypotaurine	-	5.00
L-Cysteine	-	-
HEPES[1]	10.00	-
Kanamycin[2]	75 mg/L	75 mg/L
BSA(mg/ml^{-1})[3]	4.0	-

Experimental studies

Effect of amino acid and ß-mercaptoetanol supplement on NCSU-23 on intergeneric canine embryo development with porcine oocyte cytoplasm recipient

Canine intergeneric embryos with porcine oocyte cytoplasm recipient cultured in different media with randomly distribution. Embryo development were examined for cleavage rate and 144 h for final development after cultured in media.

Results

In this experiment, amino acid and ß-mercapoethanol addition in medium NCSU-23 have no effect on embryo development (Tabel 2). The development rate of embryo until 16 cell stage in NCSU and NCSU supplement are 4.67% and 4.67 respectively and morula stage are 3.73% and 4.67%.

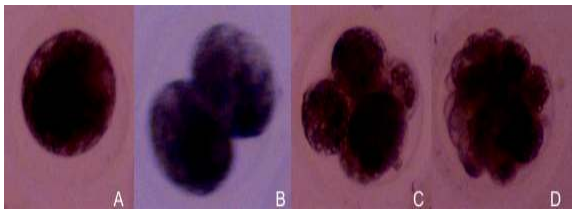

Figure 2. Development of intergeneric cloned embryo (zygote / 1 cell (A); 2 cell (B); 6 cell (C), morula (D) using porcine oocyte recipient.

Discussion

The aim of this study was wanted to learn about good supporting media culture for optimal development of intergeneric cloned dog embryo using porcine oocyte as cytoplasm donor. In this study we used NCSU-23 or NCSU-23 plus ßME and amino acid.

The supplementation of amino acids to a culture medium significantly improved the development of hamster (Schini and Bavister, 1988), mouse (Lane and Gardner, 1994; Lamb and Leese, 1994; Lane and Gardner, 1997), and cow embryos (Rosenkrans and First; 1994; Bavister and Arlotto, 1990; Bavister and Mckiernan, 1993). It has also been suggested that *in vitro* produced embryos should be exposed to amino acids as early as the oocyte stage, as this increases oocyte maternal mRNA levels and promotes preimplantation development (Watson *et al.*, 2000).In support of this opinion, it is notable that a brief exposure of zygotes to amino acid-free conditions depresses their developmental capacity and blastocyst cell numbers (Gardner and Lane,1996).

In bovine, a major step toward ameliorating media for culture embryos was discovery that the addition of Eagle's amino

acids improved embryo development (Takahashi and First, 1992; Kim *et al.*, 1993; Gardner 1994; Rosenkrans and First; 1994). Much of understanding of the way that amino acids affect mammalian embryo development and subsequent viability has come from studies on the hamster (Carney and Bavister, 1987; Bavister and Arlotto, 1990; Bavister and Mckiernan, 1993; Mckiernan *et al.*, 1995), mouse (Mehta and Kiessling, 1990; Gardner and Lane, 1993; 1996; Lane and Gardner, 1994; 1997; 1997a), and rat (Zhang and Armstrong, 1990). These studies have determined that amino acids can be either stimulatory or inhibitory to embryo development in vitro and that the presence of amino acids in culture media has a significant effect on the viability of embryos and postimplantation development.

On the other hand, low molecular weight thiol compounds, such as b-ME, have been reported to reduce cystine to cysteine and also to promote the uptake of cysteine-enhancing glutathione synthesis (Takahashi *et al.*, 2002). Moreover, it has been reported that b-ME transports cystine, forming a mixed disulphide that is taken up to facilitate the uptake of cysteine into the cells (Ishii *et al.*, 1981). Data on the effect of the concentration of b-ME are conflicting. In a study of Abeydeera *et al.* (1998) reported that adding 0 to 50 µM b-ME during in vitro maturation of pig oocytes increased intracellular glutathione concentration and subsequent embryo development and blastocyst cell numbers. The concentration (10 µM) of b-ME used in the present study was different from that (50 µM) reported by Takahashi *et al.*, (1993) but it was the same as that reported by Hamano *et al.*, (1997) (10 to 50 mM). Glutathione has many important functions in cells or embryos for the mechanism of cell defense during oxidative stress and toxicants (Gardner *et al.*, 2000). It is possible that GSH synthesis in embryos was increased by addition both of b-ME and AA into NCSU-23 medium and will be resulted in higher blastocysts and total cell numbers than that of control group.

Amino acids and b-ME both can play a vital role for preventing the oxidative stress of embryo (Ishii *et al.*, 1981; Issels *et al.*, 1988; Hamano *et al.*, 1994; Reed, 1994; Gardner and Reed, 1995; 1995a; Lane and Gardner, 1997; Takahashi *et al.*, 2002). There is a positive correlation between glutathione (GSH) and oxidative stress of the embryo. Glutathione is a tripeptide thiol synthesized by glutamic acid, cysteine, and glycine in pathway of the glutamyl cycle (Abeydeera *et al.*, 1998; Gardner *et al.*, 2000). Most cells do not take GSH intact from outside the cell; instead, GSH is broken down at the cell membrane, the constituent AA are taken up, and GSH is resynthesized inside the cell or the AA are used for other pathways (Reed, 1994). It has been reported that during IVC, GSH level drops approximately ten fold beginning from the unfertilized oocyte to blastocyst stage in mouse (Gardner and Reed, 1994; 1995). The availability of precursor AA is a regulatory factor in GSH synthesis, and it is likely that AA supplied from outside the cell provide a control point in mammalian cells (Issels *et al.*, 1988). It has been reported that nonessential AA and glutamine accelerate the time of the first three cleavage divisions and increase the time of the first three cleavage divisions and increase the time of compaction in the mouse when added to culture medium (Lane and Gardner, 1997). In another findings observed that NEAA improves development during cleavage, whereas EAA supports development after 8 cell stage (Van Winkle and Dickinson, 1995)

Unfortunately, the in vitro embryo culture media used in this study does not

supported the intergeneric embryo until blastocyst stage development, just a few morulla stages development and there are 16 block development problem, although the media used for IVC are widely used for IVC for various mammalian species (Wen *et al*., 2003). In this study we used media which usually we used as donor cytoplasm (NCSU-23) and also subsequent development is dependent on successful transfer of control to the embryonic genome, which may be deficient in embryos produced by somatic nuclear transfer. Establishment embryos from different mammalian species require species-specific embryo culture conditions (Leibfried-Rudledge *et al*., 1997). The ability of NCSU to support embryonic development of interspecies units may be attributable to the fact that this initial development of embryo is driven by recipient cytoplasm, and also thus any media supporting bovine and porcine embryo development might also support the initial development of interspecies NT embryos. Another morphological features that characterize the time of the transition is a developmental block in non permissive in vitro culture conditions (Crosby *et al*., 1988), embryos reconstructed by SCNT are more susceptible to suboptimal culture condition than IVP embryos, indicating a need to improve culture formulations to enhance development of these compromised embryos which may have abnormal metabolic activities (Chung *et al*., 2002).

It means that embryo from different mammalian species require species-specific culture media. Yong *et al*. (2003) demonstrated that the interspecies nuclear transfer embryo (macaca fibroblast cell and rabbit oocyte) have been block at 4 cell development stage when used media for rabbit embryo culture and reached the blastocyst stage when culture in media which matched with macaca embryo. Another research with Canine cell with bovine oocyte recipient in SOF medium got 0.4% blastocyst although the number of blastocyst cell only 50 (Murakami *et al*., 2005).

These indication told that, for in vitro culture canine embryo, NCSU-23 are suboptimal for embryo development although for several mammalian these media supported the development (Rosenkrans, 1998; Biggers, 2000) and improvement culture system for the development of canine embryo still necessary.

Table 2. Effect of amino acid and â-mercaptoetanol supplementation in NCSU-23 medium on canine embryo development with porcine oocyte cytoplasm recipient

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